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Initiation/Promotion Assay for Chemical Carcinogens in Rat Liver

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16. ABSTRACT
A short-term initiation/promotion bioassay has been developed in rat liver using putative preneoplastic foci as the endpoint for the detection of chemical carcinogens. The two protocols of the bioassay used in this study were varied according to the time 2/3 partial hepatectomy was performed in relation to when the initiator or test substance was given. After 7 weeks of promotion with phenobarbital in the drinking water, the rats were killed and the liver was sectioned, stained, and scored for gamma-glutamyl transpepitdase (GGTase)-positive foci. The appearance of GGTase-positive foci was taken as an indication of initiation of carcinogenesis by the chemical. Acetylaminofluorene (AAF), aflatoxin B_1 (AFB_1), benzo(a)pyrene (BaP), diethylnitrosamine (DENA), 7,12-dimethylbenzo(a)anthracene, (DMBA), dimethylhydrazine (DMH), dimethylnitrosmine (DMN), and urethane were positive in the assay, while benzene and N-methyl-N-nitro-N-nitrosoguanidine (MNNG) were negative. Phenobarbital promoted the response of AAF, AFB_1, BaP, DENA, DMBA, DMH, and DMN. Partial hepatectomy 24 h prior to initiation increased the response of AAF, BaP, DENA, DMBA, and DMH. The initiation/promotion assay in rat liver which includes (1) partial hepatectomy at 24 h prior to administering the test chemical; (2) phenobarbital promotion, and (3) GGTase-positive foci as an indication of carcinogenic activity: appears to be sensitive to a wide variety of chemical carcinogens.

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JOURNAL ARTICLE:

INITIATION/PROMOTION ASSAY FOR CHEMICAL CARCINOGENS IN RAT LIVER

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SUMMARY

A short-term initiation/promotion bioassay has been developed in rat liver using putative preneoplastic foci as the endpoint for the detection of chemical carcinogens. The two protocols of the bioassay used in this study were varied according to the time 2/3 partial hepatectomy was performed in relation to when the initiator or test substance was given. After 7 weeks of promotion with phenobarbital in the drinking water, the rats were killed and the liver was sectioned, stained and scored for gamma-glutamyl transpeptidase (GGTase)-positive foci. The appearance of GGTase-positive foci was taken as an indication of initiation of carcinogenesis by the chemical. Acetylaminofluorene (AAF), aflatoxin B_1 (AFB_1), benzo(a)pyrene (BaP), diethylnitrosamine (DENA), 7,12-dimethylbenzo(a)anthracene, (DMBA), dimethylhydrazine (DMH), dimethylnitrosamine (DMN) and urethane were positive in the assay, while benzene and *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) were negative. Phenobarbital promoted the response of AAF, AFB_1, BaP, DENA, DMBA, DMH and DMN. Partial hepatectomy 24 h prior to initiation increased the response of AAF, BaP, DENA, DMBA and DMH. The initiation/promotion assay in rat liver which includes (1) partial hepatectomy at 24 h prior to administering the test chemical; (2) phenobarbital promotion, and (3) GGTase-positive foci as an indication of carcinogenic activity: appears to be sensitive to a wide variety of chemical carcinogens.

INTRODUCTION

An initiation/promotion bioassay in rat liver has been proposed for the assessment of the carcinogenic activity of chemical and environmental samples [5, 6] Two protocols of the rat liver initiation/promotion bioassay were evaluated in

Abbreviations: AAF, acetylaminofluorene; AFB_1, aflatoxin B_1; BaP, benzo(a)pyrene; DENA, diethylnitrosamine; DMBA, 7,12-dimethylbenzo(a)authracene; DMH, dimethylhydrazine; DMN, dimethylnitrosamine; GGTase, gamma-glutamyl-transpeptidase; MNNG, *N*-methyl-*N* -nitro-*N*-nitroso-guanidine.

this study. Both included the administration of the test substance followed by 7 weeks of phenobarbital promotion and varied according to when partial hepatectomy was performed (Fig. 1). An increased incidence of GGTase-positive foci, putative precancerous lesions, was used to indicate the carcinogenic activity of the test substance. In the broad-spectrum protocol, partial hepatectomy was performed 24 h prior to the administration of the test chemical. Partial hepatectomy prior to initiation has been shown to increase the incidence of foci, hepatomas and hepatocellular carcinomas [3–7]. In the two-stage protocol, partial hepatectomy was performed 14 days after the administration of the test chemical to determine the intrinsic hepatocarcinogenic activity of the chemical. Each protocol has certain theoretical advantages: the broad-spectrum protocol should detect both hepatic and non-hepatic carcinogens, and the two-stage protocol should determine whether a compound is a hepatic carcinogen.

The sensitivity of the two protocols to hepatic and non-hepatic chemical carcinogens was determined. The chemical carcinogens tested were AAF, AFB_1, benzene, BaP, DENA, DMBA, DMH, DMN, MNNG and urethane. Two non-carcinogenic analogs were also tested: aflatoxin B_2 and benzo(e)pyrene.

MATERIALS AND METHODS

Materials

DENA and BaP were purchased from Eastman Kodak Co. (Rochester, NY); AAF, DMBA, AFB_1, aflatoxin B_2 and benzo(e)pyrene from Sigma Chemical Co., (St. Louis, MD); urethane from MCB (Gibbstown, NJ); 1,2-DMH, DMN, and MNNG from Aldrich Chemical Co. (Milwaukee, WI); benzene (glass-distilled) from Burdick and Jackson Laboratories, Inc. (Muskegon, MI); tricaprylin from ICN Nutritional Biochemicals (Cleveland, OH); sodium phenobarbital from J.T. Baker (Phillipsburg, NJ); and *N*-gamma-L-glutamyl-4-methoxy-2-naphthalamide from Polysciences, Inc. (Warrington, PA).

Experimental protocol

Male Sprague–Dawley rats (175–200 g) were purchased from Charles River (Portage, MI) and were administered by gavage the test chemical either 24 h after 2/3 partial hepatectomy in the broad-spectrum protocol or 14 days prior to 2/3 partial hepatectomy in the two-stage protocol (Fig. 1). 1 week after administration of the test substance, the rats began to receive 500 ppm sodium phenobarbital in their drinking water, ad lib. In the two-stage protocol the concentration of the phenobarbital was reduced to 100 ppm after surgery. After 7 weeks of promotion the rats were killed by decapitation and the right lateral liver lobe was quickly excised. Four tissue blocks, each approx. $10 \times 10 \times 2$ mm were taken and frozen in O.C.T. compound (Fisher, Scientific Co., Pittsburgh, PA) on dry ice. The slices were arranged so that the leading face of each block was not serially related to the leading

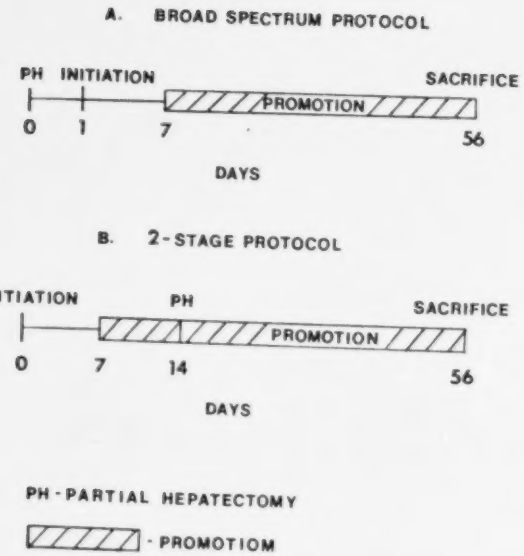

Fig. 1. Protocols of the short-term bioassay for chemical carcinogens in rat liver. Each chemical was assayed in the broad-spectrum protocol consisting of partial hepatectomy 24 h prior to the administered chemical and in the 2-stage protocol in which partial hepatectomy was performed 14 days after the administration of the chemical.

face of another block. The blocks were stored at −80 °C for up to 6 months. The tissue blocks were sliced into 6-μm sections at −25 °C. The sections were stained by the procedure of Rutenburg [8] for GGTase activity and counterstained with hematoxylin. Under light microscopy GGTase-positive foci appear as symmetrical maroon-colored areas composed of normal hepatocytes (Fig. 2). All foci which contain 9 or more nuclei were counted. For each animal at least 2 cm^2 of tissue (only one section per block of tissue) were scanned for foci.

RESULTS

Table I lists the results for the various chemicals tested in both protocols. All chemical carcinogens except benzene and MNNG were detected. A lower dose of AFB_1 and of DMN was used in the broad-spectrum protocol because when the higher dose of either chemical was administered 24 h after partial hepatectomy it resulted in a high mortality rate. AAF, BaP, DENA, DMBA, and DMH exhibited an increase in the incidence of foci when administered after partial hepatectomy in the broad-spectrum protocol when compared to their administration before partial hepatectomy in the two-stage protocol. None of the carcinogens, whether hepatic or

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TABLE I

INDUCTION OF GGTase-POSITIVE FOCI BY CHEMICAL CARCINOGENS

Chemical	Dose (mmol/kg)	N	Protocol[a]		Pheno-barbital	Foci/cm^2 (Y + S.E.)
			Broad-spectrum	2-Stage		
Carcinogen						
Acetylaminofluorene	0.3	10	+		+	4.20 ± 0.59
	0.3	10	+		–	2.29 ± 0.49
	0.3	10		+	+	2.27 ± 0.42
	0.3	10		–	–	0.77 ± 0.37
Aflatoxin B_1	0.001	8	+		+	2.38 ± 0.35
	0.001	9	+		–	0.26 ± 0.09
	0.01	7		+	+	4.15 ± 0.94
	0.01	8		–	–	1.05 ± 0.39
Benzene	30	10	+		+	0.75 ± 0.21
	30	10	+		–	0.61 ± 0.20
	30	10		+	+	0.86 ± 0.26
	30	9		–	–	0.20 ± 0.14
Benzo(a)pyrene	0.3	9	+		+	4.77 ± 0.81
	0.3	10	+		–	3.93 ± 1.09
	0.3	9		+	+	2.77 ± 1.00
	0.3	10		–	–	0.22 ± 0.13
Diethylnitrosamine	0.3	12	+		+	18.82 ± 4.63
	0.3	10	+		–	8.73 ± 1.40
	0.3	11		+	+	3.92 ± 0.91
	0.3	12		–	–	0.65 ± 0.30
Dimethylbenzo(a)anthracene	0.3	9	+		+	19.19 ± 1.48
	0.3	10	+		–	3.28 ± 0.65
	0.3	6		+	+	4.91 ± 0.50
	0.3	9		–	–	4.68 ± 0.87
Dimethylhydrazine	0.3	9	+		+	4.62 ± 0.91
	0.3	10	+		–	2.74 ± 0.48
	0.3	7		+	+	3.54 ± 0.70
	0.3	10		–	–	1.22 ± 0.32
Dimethylnitrosamine	0.03	9	+		+	1.49 ± 0.34
	0.03	10	+		–	0.56 ± 0.20
	0.3	10		+	+	4.22 ± 0.61
	0.3	10		–	–	0.96 ± 0.39
N-methyl-*N*-nitro-*N*-nitrosoguanidine	1.0	9	+		+	1.14 ± 0.20
	1.0	10	+		–	1.37 ± 0.37
	1.0	10		+	+	0.90 ± 0.33
	1.0	10		–		0.97 ± 0.42

←

Fig. 2. Gamma-glutamyl transpeptidase–positive focus. The liver section was stained for GGTase activity and counterstained with hematoxylin (× 100).

TABLE I

INDUCTION OF GGTase-POSITIVE FOCI BY CHEMICAL CARCINOGENS

Chemical	Dose (mmol/kg)	N	Protocol[a]		Pheno-barbital	Foci/cm^2 (Y + S.E.)
			Broad-spectrum	2-Stage		
Urethane	0.33	10	+		+	1.51 ± 0.45
	0.33	9	+		−	1.85 ± 0.57
	0.33	9		+	+	2.47 ± 0.37
	0.33	10		−	−	2.84 ± 0.25
Non-Carcinogenic Analogs						
Aflatoxin B_2	0.01	10	+		+	0.81 ± 0.22
	0.01	9		+	+	0.31 ± 0.16
Benzo(e)pyrene	0.3	10	+		+	0.42 ± 0.20
	0.3	7		+	+	0.09 ± 0.06
Control						
Corn oil		8	+		+	0.18 ± 0.10
		8		+	+	0.18 ± 0.18
Tricaprylin		10	+		+	0.85 ± 0.29
		10		+	+	0.38 ± 0.09
Water		9	+		+	0.52 ± 0.23
		8		+	+	0.18 ± 0.06

[a] (+) or (−) under the protocol indicates that that was the protocol used.
(+) indicates that 2/3 partial hepatectomy was performed at the time designated in that protocol.
(−) indicates that a sham operation was performed at the time designated for partial hepatectomy in that protocol.

non-hepatic carcinogens, were positive in only one of the two protocols. In the broad-spectrum protocol, phenobarbital promoted the response of 2-AAF, AFB_1, BaP, DENA, DMH, and DMN. The two non-carcinogenic analogs, aflatoxin B_2 and benzo(e)pyrene were not detected as carcinogens by either protocol.

DISCUSSION

Short-term bioassays for chemical carcinogens in rat liver have been reviewed that employ initiation/promotion formats [4, 6, 7]. Similar to the initiation/promotion bioassay originally described for mouse skin [1], these bioassays in rat liver provide for the administration of the test substance in a single dose followed by a prolonged regimen of a promoter. The promotion regimen which we employed was 500 ppm sodium phenobarbital in the drinking water for 7 weeks. This regimen of phenobarbital increased the incidence of GGTase-positive foci present 8 weeks after initiation by the various chemical carcinogens. An increase in the incidence of GGTase-positive foci, putative preneoplastic lesions [4–7], was taken to indicate

carcinogenic activity of the test substance. Foci, rather than nodules or tumors, were used as the endpoint to decrease the time requirement and to increase the sensitivity of the bioassay. Partial hepatectomy prior to the administration of the test compound in contrast to partial hepatectomy on day 14 was also demonstrated to increase the sensitivity of the assay to both hepatic and non-hepatic carcinogens including 2-AAF, BaP, DMBA, DENA and DMH. However, both protocols were sensitive to the non-hepatic carcinogens BaP, DMBA and DMH. In conclusion, a bioassay protocol consisting of partial hepatectomy 24 h prior to the administration of the test substance followed by 7 weeks of phenobarbital promotion appears to be the more sensitive of the two protocols evaluated as a short-term bioassay for chemical carcinogens.

The usefulness of a bioassay to confirm the carcinogenic activity of complex mixtures or environmental samples requires that the assay be sensitive to many different chemical classes of carcinogens [2]. Because the chemical composition of most environmental samples is unknown, it is not possible to predict beforehand which short-term initiation/promotion bioassay will be successful in confirming the presence of carcinogens. Therefore, the greater the number of chemical classes of carcinogens detected by an assay, the greater the probability that carcinogenic activity will be successfully confirmed. The rat liver initiation/promotion assay detected representatives of the following classes of chemicals; aromatic amines (2-AAF), direct-acting alkylating agents (DMH, urethane), fungal toxins (aflatoxin B_1), nitrosamines (DENA, DMN), and polycyclic aromatic hydrocarbons (BaP, DMBA). Benzene and MNUG were the only carcinogens not detected. The non-carcinogenic analogs aflatoxin B_2 and benzo(e)pyrene were also not detected. Thus the rat liver initiation/promotion bioassay appears to be an assay suitable for use in the confirmation of the carcinogenic activity of chemicals and environmental samples.

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